PB86-162369

Use of a Human/Mouse Hybrid Cell Line to Detect Aneuploidy Induced by Environmental Chemicals

(U.S.) Health Effects Research Lab.
Research Triangle Park, NC

1985

U.S. Department of Commerce
National Technical Information Service
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TECHNICAL REPORT DATA
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1. REPORT NO. EPA/600/J-85/322	2.	3. PB86-162369
4. TITLE AND SUBTITLE: Use of A Human X Mouse Hybrid Cell Line To Detect Aneuploidy Induced By Environmental Chemicals (Journal Version)		5. REPORT DATE: 1985 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S): Raghbir S. Athwal and Shahbeg S. Sandhu		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Department of Microbiology, New Jersey Medical School, 100 Bergen Street, Newark, NJ 07103		10. PROGRAM ELEMENT NO. ACSL1A 11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS: Health Effects Research Laboratory, Office of Research and Development, US Environmental Protection Agency, Research Triangle Park, NC 27711		13. TYPE OF REPORT AND PERIOD COVERED: Journal Article 14. SPONSORING AGENCY CODE: EPA-600/11
15. SUPPLEMENTARY NOTES: Mutation Research 149 (1985) 73-81 (Feb.)		

16. ABSTRACT

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17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

18. DISTRIBUTION STATEMENT: RELEASE TO PUBLIC	19. SECURITY CLASS (This Report): UNCLASSIFIED	21. NO. OF PAGES: 10
	20. SECURITY CLASS (This page): UNCLASSIFIED	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

EPA/600/J-85/322
JOURNAL ARTICLE

(FE3)

Mutation Research, 149 (1985) 73–81
Elsevier

MTR 03988

Use of a human × mouse hybrid cell line to detect aneuploidy induced by environmental chemicals

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(Received 25 July 1984)
(Revision received 10 September 1984)
(Accepted 17 September 1984)

Summary

A short-term assay utilizing a human/mouse monochromosomal hybrid cell line R3-5, to detect chemically induced aneuploidy in mammalian cells is described. A single human chromosome transferred into mouse cells was used as a cytogenetic marker to quantitate abnormal chromosome segregation following chemical treatment. The human chromosome present in the mouse cells can be readily identified by differential staining procedures. The frequency of cells containing 0 or 2 human chromosomes in the progeny of chemically treated monochromosomal hybrid cells provided a direct measure of aneuploidy. We tested the sensitivity of the proposed system with 3 model chemicals (colcemid, cyclophosphamide and benomyl) known to induce numerical or structural changes in chromosomes. The frequency of an abnormal segregation of the human chromosome was found to be dose dependent and consistently higher than controls. This system has the capability to detect gain as well as loss of a chromosome resulting from nondisjunction or other mechanisms leading to aneuploidy.

Over the past 20 years, considerable effort has been expended to develop short-term assay systems for the identification of genotoxic agents in our environment. The research has primarily been focused on the detection of genotoxins, which interact with DNA to produce gene mutations in bacteria (McCann and Ames, 1977) or chromosome damage in mammalian cells (review in Sugimura et al., 1982). However, some chemicals may not change DNA or chromosomes but interfere with the division process of cells to produce numerical changes (aneuploidy) in the cell genome (Sawada and Ishidate, 1978; Weber et al., 1976; Liang et al., 1983; Tsutsui et al., 1983). Aneuploidy as an end point in chemical mutagenesis has been emphasized relatively recently and only a limited amount of work has been reported in this direction (Zimmermann et al., 1979). Some studies have been conducted utilizing insects (Liang et al., 1983; Zimmering, 1982) but lower eukaryotes have been employed most commonly to detect chemically induced aneuploidy (Mir and Wright, 1978; Morpurgo et al., 1979; Kappas, 1978; de Bertoldi and Griselli, 1980; Sora et al., 1982). However, mitotic poisons may affect cells differently in higher and lower eukaryotes (Haber et al., 1972) and this difference has been demonstrated to be due to the presence of different target sites on the microtubules (Heath, 1975). Therefore, information obtained from a test system based on mam-

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malian cells would be more relevant than a non-mammalian system for the estimation of risk to human health.

In this paper, we propose a mammalian cell culture system using a human/mouse somatic cell hybrid containing a single human chromosome (monochromosomal hybrid) to quantitate chemically induced aneuploidy. The human chromosome present in mouse cells serves as a cytogenetic marker and can be easily identified by differential staining procedures. Progeny of a monochromosomal hybrid would normally contain a single human chromosome. The cells containing two or no human chromosomes would result from gain or loss of the human chromosome either by nondisjunction, or other mechanism. We have used the segregation pattern of the human chromosome following the exposure of cells to the test chemical to assess the potential of a chemical to induce aneuploidy. Three chemicals, colcemid, cyclophosphamide and benomyl, were used as model compounds to assess the utility of the proposed test system.

Materials and methods

Cell line and growth conditions

A microcell hybrid (R3-5) between mouse and human cells containing a single human chromosome (monochromosomal hybrid) was used in these experiments. The human chromosome present in R3-5 was transferred to mouse cells (RAG) deficient for hypoxanthine–guanine phosphoribosyl transferase (hprt$^-$), by a two-step process involving gene transfer and microcell fusion techniques (Dhar et al., 1984). A dominant selectable marker, *E. coli* xanthine–guanine phosphoribosyl transferase (Ecogpt) gene cloned into a plasmid (Mulligan and Berg, 1981), was first transferred to human MGH-1 cells to produce cell lines each carrying Ecogpt integrated into a different human chromosome. Microcells prepared from one of these cell lines were fused with RAG cells and microcell hybrids were isolated by selection for growth in the medium containing mycophenolic acid and xanthine (R.S. Athwal, unpublished results). Following cytogenetic analysis, a microcell hybrid R3-5 containing a single human chromosome was chosen for the studies described in this report. The human chromosome present in R3-5 thus carries a dominant selectable marker (Ecogpt) and was identified to be chromosome 2. Cultivation of these cells in the medium containing mycophenolic acid and xanthine (MX), therefore assures the presence of the human chromosome 2 in every cell. The cells which may have lost the human chromosome would not survive in the selective medium. The cell cycle time of these cells estimated from the doubling time by cell count was determined to be 18–20 h.

R3-5 cells were routinely cultured at 37°C in 10% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (DME) containing mycophenolic acid and xanthine (MX medium). Although growth in the selective medium assured that all the cells contained the transferred human chromosome, the cell line was periodically analyzed cytologically for confirmation.

For each experiment 0.5×10^6 cells were plated in T25 flasks in nonselective medium (DME) 24 h prior to the chemical treatment. Following growth for 24 h, the cultures were rinsed twice with PBS and fed with DME containing the test chemical. After exposure to the chemical for 24 h, cultures were rinsed with PBS × 2, refed with fresh DME, allowed to grow for another 24 h and then harvested for cytogenetic analysis.

Cytogenetic analysis

Following exposure to the test chemical, cells were arrested in mitosis with colcemid (0.2 μg/ml) for 2 h. Mitotic cells harvested by shaking the flasks were swollen in hypotonic solution (0.075 M KCl) for 15 min and then fixed in 3 changes of fixative (3 methanol : 1 acetic acid). Metaphase spreads prepared by air drying were stained either by Giemsa-11 (G-11) method (Bobrow and Gross, 1974) or with Hoechst 33258 (Hilwig and Gropp, 1972) to distinguish between human and mouse chromosomes.

For G-11, 1-day-old slides were soaked in water at 37°C for 30 min and then rinsed in 0.05 M sodium phosphate (Na_2HPO_4) buffer (pH 11.3) for 1 min. Following pretreatment, slides were stained in the staining solution prepared in Na_2HPO_4 (0.05 M) buffer (pH 11.3) for 3–4 min at 37°C. The staining solution was prepared fresh by mixing 240 μl of Azur B (1% in H_2O) and 200

μl of eosin (0.25% in methanol) into 20 ml of buffer.

Alternatively, in some experiments the metaphase spreads were stained with the fluorescent dye, Hoechst 33258. The slides were pretreated with NaOH (0.07 N) dissolved in 30% ethanol for 1–2 min and rinsed 3 times in 70% ethanol to stop denaturation. Following the ethanol treatment, the slides were rinsed twice in PBS and stained for 15 min in Hoechst 33258 (0.05 μg/ml) dissolved in PBS. The stained slides were rinsed with distilled water, air dried, and wet mounted in 0.08 M sodium phosphate, 0.12 M sodium citrate buffer (pH 4.1). The metaphase spreads were examined using a fluorescence microscope (Zeiss) equipped with an epilluminator to give excitation and reflector wave lengths of 365 nm and 420 nm, respectively.

Chemicals and concentrations

In these experiments, we used 3 chemicals as model agents: colcemid, cyclophosphamide, and benomyl. Treatment concentrations (Table 1) for each chemical were selected on the basis of toxicity. Toxicity was determined by exposing 10^6 cells, seeded in T25 flasks, for 24 h to different concentrations of a chemical. The inhibition of growth, determined by counting viable cells following chemical treatment for 24 h was used as a measure of toxicity. The concentrations tested for each chemical included at least 1 dose above and 1–3 doses below the 50% toxicity level. Since the toxicity of a chemical is influenced by cell density at the time of treatment, conditions similar to those used for the induction of aneuploidy were used for the toxicity analysis.

Colcemid. Colcemid was purchased as a 10 μg/ml solution in Hanks' BSS (Gibco) and diluted further in the medium. The concentration that inhibited cell multiplication by 50% was determined to be 0.02 μg/ml, and the concentrations used in the experiments were 0.005, 0.01, 0.02, 0.05, and 0.1 μg/ml.

Cyclophosphamide. Cyclophosphamide (Sigma Cat. No. C0768) was dissolved in water at a concentration of 1 mg/ml. A concentration of 15 μg/ml of cyclophosphamide was found to give 50% inhibition of growth in R3-5 cells. The concentrations of 10, 15 and 20 μg/ml were used in the experiments. Cyclophosphamide requires metabolic activation and thus was used both in combination with S9 fraction of rat liver microsomes and without metabolic activation.

Benomyl. Benomyl (Du Pont de Nemours, Wilmington, DL, U.S.A.) was dissolved at a concentration of 3 mg/ml in dimethyl sulfoxide (DMSO) and diluted further in 50% DMSO. A concentration of 7.5 μg/ml of benomyl was found to produce 50% toxicity. The concentrations of 1.5, 3, 7.5, and 15 μg/ml were tested for the induction of aneuploidy in R3-5 cells.

Results

Cytogenetic analysis

After the chemical treatment, individual metaphases were scored to estimate the total number of chromosomes and to determine the number of human chromosomes present in each cell. The total number of chromosomes present in R3-5 cells varies between 50 and 60 (Fig. 1). Although R3-5 cells are not true diploids, to simplify the data cells containing the parental chromosome complement (50–60 chromosomes) were considered as diploids, while cells with twice or more that number of chromosomes were classified as polyploids (Fig. 1). Only the cells containing the diploid number of chromosomes were scored for the presence of human chromosomes (Table 1). Since R3-5 cells contained only a single human chromosome, the presence of 2 or 0 human chromosome(s) was considered as evidence for aneuploidy resulting from nondisjunction or loss of chromosome (Table 1).

With G-11, human chromosomes are stained blue and have magenta centromeres, whereas mouse chromosomes stain magenta with blue centromeres (Fig. 1). Any human chromosome present in mouse cells can therefore be identified by difference in color. In the case of Hoechst 33258, a human chromosome present among mouse chromosomes is readily identified by the lack of characteristic centromeric fluorescence of the mouse chromosomes (Fig. 1). In addition, chromosome 2, being morphologically different from mouse chromosomes, further facilitated the identification (Fig. 1).

The data on the number of cells containing 1, 2 and 0 human chromosomes following chemical

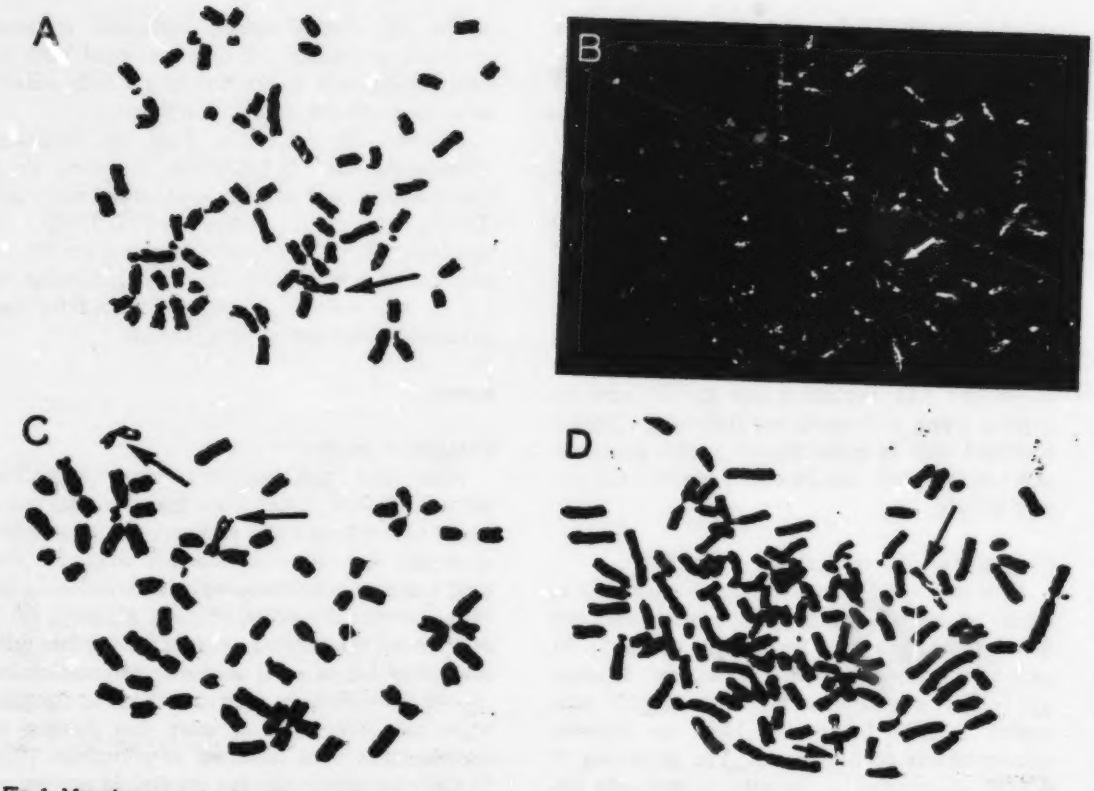

Fig. 1. Metaphase spreads of monochromosomal hybrid R3-5. (A) Stained with G-11. Single human chromosome present in mouse cells is distinguishable by the color difference in the chromosomes of the two species. (B) Stained with Hoechst 33528. Human chromosome is identified by the lack of intense centromeric fluorescence, characteristic of mouse chromosomes. (C) Metaphase of R3-5 cell with diploid number of chromosomes containing 2 human chromosomes following benomyl treatment. (D) Metaphase spread of a polyploid cell containing 2 human chromosomes obtained by colcemid treatment.

treatment are compiled in Table 1. The relative frequencies of aneuploid and polyploid cells for each chemical are plotted in Fig. 2. The results obtained with each chemical are as follows:

Colcemid. Colcemid binds to tubulin and inhibits its polymerization to form microtubules (Murgolis and Wilson, 1977) and the effect is reversible when the drug is removed (Brinkley et al., 1967). However, reorganization of microtubules after the removal of the drug may lead to an abnormal segregation of chromosomes and consequently result in aneuploid or polyploid cells. In our study, the frequency of aneuploid cells increased with an increase in the concentration of the drug up to 0.05 μg/ml, and the increase was linear up to a concentration of 0.02 μg/ml (Fig. 2A). However, the number of aneuploid cells was relatively lower at 0.1 μg/ml than with the number obtained at 0.05 μg/ml (Fig. 2A, Table 1).

In comparison with the control, no increase in the frequency of polyploid cells was observed up to a concentration of 0.01 μg/ml. However, the number of polyploid cells increased considerably at 0.02, 0.05 and 0.1 μg/ml. An increase in the frequency of polyploid cells accompanied a decrease in the frequency of aneuploid cells at 0.1 μg/ml (Fig. 1A). At the same time, at high concentration of colcemid with an increase in the number of polyploid cells in the population, there was a corresponding increase in the ratio of poly-

TABLE 1

NUMBER OF CELLS CONTAINING 1, 2 OR 0 HUMAN CHROMOSOMES AND POLYPLOID CELLS FOLLOWING EXPOSURE TO THE TEST CHEMICALS

Chemicals and conc. (μg/ml)	Number of metaphases examined	Cells with 1, 2, and 0 human chromosomes			Number of aneuploid cells (A)	Number of polyploid cells (P)	Aneuploid cells [a] (%)	Ratio P/A [b]	Cells [c] with chromosome aberrations (%)
		1	2	0					
Colcemid									
0	200	191	4	4	8	1	4.02	0.12	0.5
0.005	200	185	7	7	14	1	7.04	0.07	0.5
0.01	200	180	9	10	19	1	9.55	0.05	0
0.02	200	139	12	14	26	35	15.75	1.11	0.5
0.05	200	96	16	20	36	68	27.27	1.25	0
0.1	200	86	11	14	25	89	22.52	1.98	0.5
Cyclophosphamide									
With metabolic activation									
0	200	191	3	5	8	1	4.02	0.12	0
10	200	190	3	6	9	1	4.52	0.11	3
15	200	183	5	11	16	1	8.04	0.06	4
20	200	182	5	12	17	1	8.54	0.06	6
Without metabolic activation									
0	200	192	4	3	7	1	3.51	0.14	0
10	200	191	5	3	8	1	4.02	0.12	0.5
15	200	192	3	4	7	1	3.51	0.14	0
20	200	193	3	3	6	1	3.03	0.16	0.5
Benomyl									
0	200	192	1	3	4	4	2.04	0.98	0.5
1.5	200	164	13	20	33	4	16.75	0.12	0.5
3.0	400	289	35	39	74	37	20.39	0.45	1
7.5	300	161	26	30	56	83	25.81	1.07	1
15	300	142	24	27	51	107	26.42	1.35	4

[a] The percentage of aneuploid cells was calculated by adding cells with 0 and 2 human chromosomes and taking diploid and aneuploid cells for total (i.e., excluding polyploid cells).

[b] Ratio of polyploid/aneuploid cells was calculated from the percentage of polyploid and aneuploid cells.

[c] Cell with chromosomal aberrations involving human chromosome were counted.

Note: Aneuploidy in this system refers to the gain or loss of the human chromosome in R3-5 cells, while polyploidy refers to the increase in the total number of chromosomes by a factor 2 or more.

ploid/aneuploid (P/A) cells (Table 1).

The data on chromosome aberrations involving human chromosome showed no difference in control and the cultures treated with colcemid (Table 1).

Cyclophosphamide. Cyclophosphamide (Cy), a commonly used antineoplastic drug, is a potent mutagen and clastogenic agent when metabolically activated (Mohn and Ellenberger, 1976; Goetz et al., 1980); it has also been reported to induce aneuploidy in mouse germ cells (Pacchierotti et al., 1983).

As given in Table 1, cyclophosphamide without metabolic activation had no effect on the segregation of the human chromosome or cell division at any concentration used. The data on Cy without metabolic activation thus provided a negative control and show that abnormal segregation of the human chromosome occurred only with potent chemicals.

With metabolic activation, Cy, at the lowest concentration used (10 μg/ml), induced no change in the frequency of aneuploid cells. However, the number of aneuploid cells almost doubled at 15 μg/ml, and no further increase occurred at 20 μg/ml (Fig. 2B). It was interesting to find that the increase in the number of aneuploid cells at higher concentrations was only in the class of cells that had lost the human chromosome (0-chromosome class), rather than the one with an extra chromosome (2 chromosomes) (Table 1). We interpret these data to show that Cy does not have an effect on cell division per se; instead the aneuploid cells in this case result from structural damage that leads to a loss of chromosomes. This interpretation is supported by the fact that the frequency of polyploid cells did not change as a result of Cy treatment (Fig. 2B). In addition, a dose-dependent increase in the frequency of cells with chromosomal aberrations involving human chromosome was observed (Table 1). At the same time, in contrast to colcemid, the ratio of polyploid/aneuploid cells decreased with an increase in concentration of Cy (Table 1).

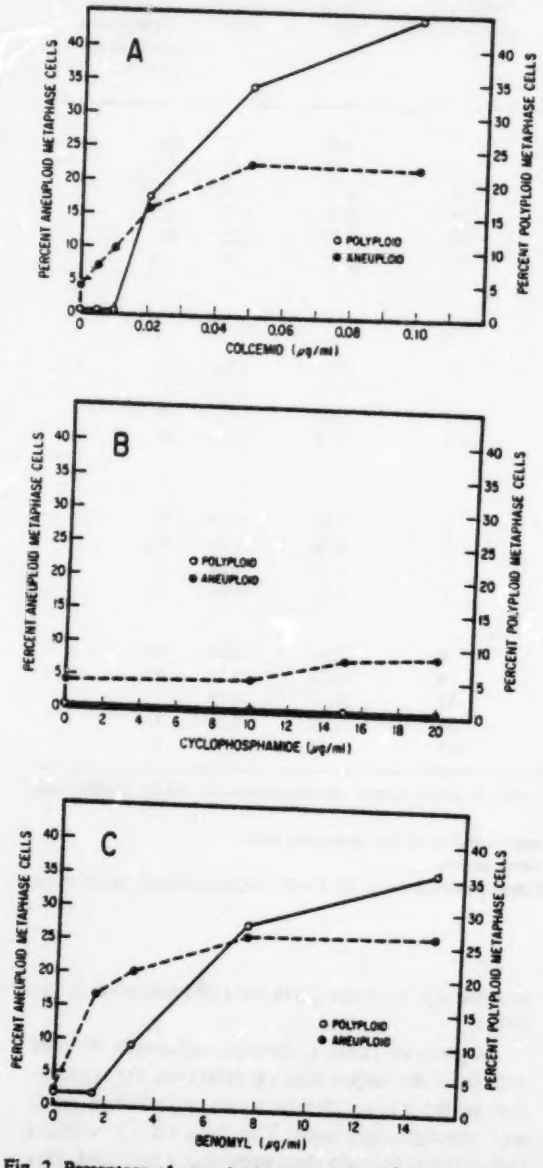

Fig. 2. Percentage of aneuploid and polyploid cells with R3-5 cultures exposed to: (A) colcemid, (B) cyclophosphamide, and (C) benomyl. The percentage of aneuploid cells was determined by adding together the cells with 0 and 2 human chromosomes.

Benomyl. Benomyl inhibits the assembly of microtubules in the same manner as colcemid, and it has previously been reported to induce aneuploidy in yeast and fungi (Welker and Williams, 1980; Morpurgo et al., 1979; Kappas, 1978) and in human lymphocytes (Tenchini et al., 1983). In our experiments, compared to the control, the frequency of aneuploid cells was higher even at the lowest concentrations used (1.5 μg/ml). A nonlinear response was observed at concentrations up to 7.5 μg/ml (Fig. 2C). No change in the frequency of aneuploid cells occurred at 15 μg/ml, in comparison to 7.5 μg/ml benomyl (Fig. 2C). At 1.5 μg/ml, the frequency of polyploid cells was found to be equivalent to the control, but a substantial increase was observed at higher doses (Table 1, Fig. 2C). At the highest concentration (15 μg/ml), although the frequency of aneuploid cells remained the same as at 7.5 μg/ml, the number of polyploid cells increased. As with colcemid, a dose-dependent increase in P/A ratio was observed (Table 1). Concentrations higher than 15 μg/ml were highly toxic and did not yield enough mitotic cells for a meaningful analysis.

Benomyl also induced breaks in the human chromosome. The frequency of cells with chromosome aberration increased with the dose. Thus, in addition to being a mitotic poison, benomyl is also a potent clastogenic agent.

Discussion

The data presented in this paper show the usefulness of the proposed system to quantitate chemically induced aneuploidy. The aneuploid condition includes both the gain ($2n + 1$) and loss ($2n - 1$) of a chromosome. The gain of a chromosome occurs due to nondisjunction, whereas the loss can result from nondisjunction or the failure of a chromosome to incorporate into the daughter nucleus by other mechanisms, such as anaphase lagging or lack of replication (Bond and Chandley, 1983).

A human chromosome present in mouse cells provides an excellent cytological marker. The morphological features (size and position of the centromere) of human chromosome 2, present in R3-5 cells, combined with differential staining make it relatively easy to distinguish this chromosome from those of mouse (Fig. 1). The proposed system is capable of detecting chromosome gain, as well as loss resulting from the effect of chemicals. The number of cells that lost the human chromosome was consistently higher than the number that showed a gain (Table 1). This occurred at all concentrations of chemicals tested (Table 1). This result is expected on the basis that more than one mechanism could account for the loss. In particular, it was very interesting to find that Cy induces only chromosome loss and no chromosome gain (Table 1). This correlates with the fact that no increase in the frequency of polyploid cells was observed to occur as a result of Cy treatment. Cyclophosphamide is a known clastogenic agent (Goetz et al., 1980), chromosome loss in this case may have occurred as a result of chromosome breakage rather than interference with cell division. Dose-dependent increase in the frequency of cells with chromosome aberrations observed in the case of Cy also suggests the existence of such a phenomenon (Table 1).

Another important feature of R3-5 cells is the presence of a selectable marker on the human chromosome. Growth of these cells in the selective medium, prior to chemical treatment assures the presence of this chromosome in every cell. The presence or loss of the human chromosome in R3-5 cells does not interfere with the growth of cells in the nonselective medium.

The other mammalian cell culture systems proposed to detect aneuploidy include the use of continuous or early passage Chinese hamster cell lines (Kato and Yoshida, 1970; Hsu et al., 1983; Onfelt and Klasterska, 1983; Danford, 1984). In these systems, complete mitotic block, total chromosome count and chromosome count combined with the analysis of abnormal anaphases have been used as criteria to assay for aneuploidy. A chromosome count on metaphases does not provide complete information on the induction of aneuploidy. A cell may have lost or gained a chromosome in two different homologous pairs and still may retain the parental chromosome number. In this case, although aneuploidy occurs, the chromosome number remains unchanged.

Another system similar to ours in principle, uses human male lymphocytes (Tenchini et al., 1983). In this system, the human Y chromosome was used as a marker to assess aberrant segregation following chemical treatment. The occurrence of YY mitosis was considered to result from a nondisjunctional event. The data obtained with this system did not show a concentration-dependent response and the frequency of YY cells was rather low. This may be due to the sensitivity of human diploid cells (lymphocytes) to the test chemicals. Thus, the lack of dose response may have been due to the elimination of cells in which the abnormal segregation of chromosomes other than Y had occurred.

In our hybrid cell system, although the effect was nonlinear, the frequency of aneuploid cells correlated with the concentration of the test chemical in all the experiments except at high doses. The frequency of polyploid cells increased in response to concentration for colcemid and benomyl (Fig. 2). At higher concentrations, an increase in the frequency of polyploid cells was accompanied by a relative decrease in the number of aneuploid cells (Fig. 2), which was reflected by a shift in polyploid/aneuploid ratio from low to high as the concentration of the test chemical increased (Table 1). We believe that as the concentration of the chemical increases more and more sites on the target molecule are occupied until saturation which leads to complete disruption of the division process. A complete, irreversible inhibition of microtubules at high concentration gives rise to poly-

ploid cells, whereas aneuploidy results from abnormal segregation during recovery following temporary inhibition of cell division. Our data show that the P/A ratio remains unaltered with a non-potent chemical, while it decreased with an increase in the concentration if aneuploidy is induced by a mechanism other than interference with the spindle formation. A dose-related change in P/A ratio thus may serve as an index for potential of a chemical to induce aneuploidy.

In conclusion, R3-5 provides a continuous cell line to detect the potential of environmental chemicals to induce aneuploidy. The selectable marker present on the human chromosome allows the selection of cells for the presence or loss of this chromosome (R.S. Athwal, unpublished results). This would make it possible to develop an assay system by selection for the detection of aneuploidy. In addition human chromosome 2 can be transferred further to any cell type that may have the ability to metabolize promutagens.

Acknowledgments

We thank Mr. M.A. Ahmad for technical assistance and Mr. S. Desai for photography. Studies included in this report were supported in part by Contract No. 4D3497 NAEX from the U.S. Environmental Protection Agency, Grant No. 1-845 from the March of Dimes Birth Defects Foundation, and Grant No. CA 28559 from the National Cancer Institute, National Institute of Health.

Although the research described in this article has been funded in part by the U.S. EPA, it does not necessarily reflect the view of the agency and no official endorsement should be inferred.

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